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Natural antisense transcripts as therapeutic targets

Paul Halley[1], **Olga Khorkova**[2], and **Claes Wahlestedt**[1]

[1]Department of Psychiatry and Behavioral Sciences, and Center for Therapeutic Innovation, University of Miami Miller School of Medicine, Miami, FL 33136, USA.

[2]OPKO-CURNA 10320 USA Today Way, Miramar, FL 33025, USA

At the turn of the 21st century, the central dogma of molecular biology was challenged by the unexpected discovery that only 1.2% of all genes transcribed from the human genome actually encode proteins. In fact, large-scale sequencing of the human and mouse genomes revealed that the other 99% is comprised of thousands of non-protein coding transcripts or ‘non-coding RNAs’ (ncRNA) that form complex and overlapping networks, and act as transcriptional and post-transcriptional regulators within various cells and tissues [1-3]. These are broadly classified, according to their sizes, into short (<200bp) and long ncRNAs (lncRNAs) [4]. Although short ncRNAs, in particular microRNAs (miRNAs), have now been extensively characterized [5], until recently less attention had been paid to the abundance of long non-coding transcripts, previously viewed by many as genomic junk or ‘dark matter’. However, observations that a significant number of lncRNAs are conserved across species, and are expressed in a regional, temporal and cell-specific manner, suggested that these transcripts could indeed have functional relevance [6]. Particularly within the last few years, lncRNAs have been attracting increasing interest, in light of growing evidence that they play key roles in a variety of cellular processes. In 2006, it was proposed that lncRNAs, such as the natural antisense transcripts (NATs), represented novel therapeutic targets to influence the expression of genes or pathways that were previously considered to be undruggable [7]. In the years since, not only have we developed a greater understanding as to how these RNA molecules exert their control, but this type of innovative thinking has also inspired the formation of new biotech companies. Here we describe the various regulatory functions of NATs, as well as their emerging links to disease, and focus on their continued promise as viable therapeutic targets.

Regulatory roles of natural antisense transcripts

Although classification and nomenclature are still in its early stages, the number of functionally annotated lncRNAs continues to rapidly expand. Indeed several subclasses have already been broadly characterized which include NATs [8], large intergenic ncRNA (lincRNAs) [9], and totally and partially intronic non-coding transcripts (TINs and PINs)

[10]. Although lincRNAs have also come to prominence in recent years, particularly due to their association with various types of cancer (table 1), a comprehensive description of the expanding fields of these and other types of lncRNAs is beyond the scope of this short review. Instead we will focus on the therapeutic potential of NATs, arguably the best characterized of the lncRNAs. In addition to the information presented here, we encourage the reader to refer to the following excellent articles, which further describe different themes within the diverse world of lncRNAs [11-14].

NATs are lncRNAs that are transcribed from the opposite DNA strand to sense (protein-coding) transcripts and overlap in part with sense RNA, sense promoter or other regulatory regions[2]. They can originate from coding or non-coding DNA, including genic, intergenic or intronic sequences, and display a number of similarities to mRNA such as 5′ capping, 3′ polyadenylation and alternative splicing [8]. The most common form of regulation seen for NATs is the pairing of one of these non-coding antisense RNAs with a partner sense transcript [2]. Results from the FANTOM3 project revealed at least 1000 sense-antisense transcript pairs that were well conserved between mouse and human, as well as many thousands considered to be non-conserved [15]. In 2010, Faghihi and colleagues examined the targeted knockdown of 797 evolutionary conserved NATs and found evidence of regulatory roles for a number of sense-antisense pairs [16]. Indeed, NATs have now been identified for a broad range of mammalian genes involved in various diverse processes such as cell growth and differentiation, development, metabolism, cardiovascular function and synaptic plasticity (tables 1 and 2). Despite being processed in much the same way as mRNAs, NATs do not all display the same characteristics. For example, they can be polyadenylated on non-polyadenylated, or be localized within the nucleus or the cytoplasm. While they have generally been shown to be less abundant than sense transcripts[17], the expression level of a given antisense transcript can also vary based on cell type [18, 19]. Using a custom ncRNA array, Clark and colleagues very recently demonstrated that the half-lives of cis-acting antisense transcripts can range from three to ten hours, although the majority of those examined clustered at about five to six hours [20]. The stability of NATs also appears to be influenced by their cellular localization, with nuclear transcripts shown to be more unstable[20].

So far, the regulatory roles of various forms of lncRNAs have generally been demonstrated through large-scale molecular screens, in which their activities have been disrupted using RNA interference technology. These studies have demonstrated that endogenous lncRNAs can act by repressing or promoting the expression of their target genes. This regulation has been seen to occur either is *cis*-, whereby the lncRNA originates within the same genetic locus as it target sense transcript, or *trans*-, whereby the lncRNA is derived at a distant chromosomal location from the gene on which it acts [16, 21]. Although they demonstrate a high degree of target specificity, the manner in which NATs regulate their corresponding paired sense transcripts is thought to be quite diverse, involving various suggested transcriptional and post-transcriptional mechanisms (for review see [8]). For example, a number of them have now been shown to interact with the promoter regions of their corresponding sense strand in *cis*-, and influence DNA methylation, or act as scaffolds for the targeted recruitment of histone modifying complexes that dictate its chromatin state [22].

Indeed the presence of RNA-binding motifs in many chromatin-modifying enzymes suggests that some NATs that are in low abundance within the nucleus can act locally to orchestrate histone modifications and thus mediate gene silencing [23]. So far, the best-characterized example of this is the recruitment of the gene-silencing polycomb recessive complex 2 (PRC2), which is known to induce trimethylation of the lysine 27 residue on histone H3 (H3K27me3), a mark of transcriptionally silent chromatin. RNA immunoprecipitation (RIP) combined with directional RNA sequencing revealed that the PRC2 complex associates with over 9000 RNAs in mouse embryonic stem cells, and that almost 3000 of these RNAs are NATs. In fact, PRC2 recruitment by one such NAT, the X (inactive)-specific transcript (Xist), can lead to the inactivation of the entire X-chromosome in humans through heterochromatinization [24], which can also have important implications for various X-linked diseases [25].

Alternatively, sequence complementarity of cis-acting NATs means that they can also regulate the expression of their paired sense RNAs through the formation of RNA duplexes, both in the nucleus and the cytoplasm. The formation of RNA-RNA duplexes between NATs and their partner sense transcript in the nucleus can lead to differential RNA splicing or reduced cellular availability of the sense RNA through nuclear retention [26, 27]. Furthermore RNA duplex formation in the cytoplasm has been shown to influence sense transcript stability through alterations in secondary structure or by masking other regulatory binding sites [18, 28]. RNA duplexes within the cytoplasm have also been shown to inhibit sense RNA translation between initiation and elongation stages [29].

Given this complex level of regulation, it is likely that altered cellular NAT expression could also contribute to a number of pathological states. This is supported by observations that some antisense transcripts, such as BACE1-AS, HSR and HIF1-AS, can be influenced by various cellular stressors [18, 30-32]. Furthermore NATs have now been identified for genes involved in various neurological conditions, such as Alzheimer's [18], Huntington's [33], and Parkinson's disease [34], as well as cardiovascular [35], and metabolic disorders [34]. Although it currently remains to be seen whether some of these transcripts are actually differentially expressed in patient populations, as we will discuss later, functional validation of these and other similar NATs may lead to the identification of novel drug targets to combat disease progression (table 1).

While a number of mutations have been reported in various ncRNA loci [3, 36, 37], there is so far very limited evidence from human genetic studies to show that mutations or single nucleotide polymorphisms (SNPs) within genes encoding NATs can affect their expression levels or give rise to disease phenotypes. However, genome-wide association studies have demonstrated that the intergenic region encoding ANRIL, a large antisense transcript that mediates the transcriptional regulation of the tumor suppressor genes INK4b/ARF/INK4a at the INK4a locus [38], is associated with increased susceptibility to cancer, type 2 diabetes and coronary disease [39]. Indeed, SNPs within the ANRIL-encoding region that are linked with increased susceptibility to atherosclerosis, are associated with reduced ANRIL expression levels in purified peripheral blood T–cells. On the other hand, ANRIL is seen to be over-expressed in prostate cancer cells, inducing the silencing of tumor suppressor genes INK4b/ARF/INK4a, through the recruitment of PRC1 [38]. A further report has shown that

ANRIL is also required for the PRC2-mediated silencing of the P15/INK4B tumor suppressor gene, and that disrupting its interaction with the PRC2 complex can inhibit cell proliferation [40]. Increased expression of a NAT for another tumor suppressor gene p15 (p15AS) was also observed in leukemia patients, which correlated with a marked decrease in p15 sense levels [41]. Here, the investigators showed that p15AS expression induced dimethylation of histone 3 lysine 4 (H3K4) and reduced dimethylation of histone 3 lysine 9 (H3K9) at the p15 promoter, resulting in persistent transcriptional silencing. While the cause of this p15AS upregulation is unknown, this is a clear example of how a differentially expressed NAT could function as a potential molecular marker for disease, as well as a novel therapeutic target.

Natural antisense transcripts as therapeutic targets

In vitro screens combining RNA interference technology with standard cell culture techniques have been invaluable in terms of functionally characterizing various types of lncRNAs. In addition, they have also demonstrated that manipulating these regulatory transcripts can result in the differential expression of their corresponding sense genes, and that this can have functional significance by driving certain types of cellular behavior [16, 21]. While reports have shown that inhibiting some NATs leads to a corresponding decrease in sense transcript levels (concordant regulation), a significant proportion of thus-far validated NATs have been seen to repress the expression of their sense counterparts (discordant regulation) (table 2). Despite the fact that as much as 70% of mammalian genes have been seen to have antisense partners [2], the potential of targeting NATs has so far been relatively unexplored. In this regard, our group has previously proposed that pharmacological strategies designed to interfere with antisense function could, in principal, be used to enhance the expression levels of a given therapeutic gene, through a de-repression of discordant regulation [7]. This type of approach was first published by Katayama and colleagues [2] and has since been demonstrated by several investigators, in various different systems (table 2).

From a therapeutic perspective, targeting functionally characterized NATs offers a number of advantages. Firstly, their highly specific mode of paired sense-antisense regulation should ensure that only a single gene is being activated (or we acknowledge, as in the case of ANRIL above, a small subset of related genes). Secondly, modulating NATs only affects the cell populations that normally express their target proteins [18, 19, 42]. For this reason, identifying and inhibiting discordantly-acting NATs would lead to the up-regulation of a protein in its natural cellular environment, suggesting that it will be correctly spliced and folded with the appropriate modifications, and therefore fully functional. This approach could be beneficial for the treatment of certain conditions, whereby enhancing gene expression might help to reverse an adverse phenotype or protect against further disease onset. For example, the selective up-regulation of specific genes involved in the reverse cholesterol transport pathway, such ABCA1, ABCG1 or APOA1 may represent a novel approach to promote high-density lipoprotein (HDL) formation and treat heart disease [43]. So far, strategies to enhance HDL have targeted the liver X receptor (LXR), as stimulation of this receptor has been shown to induce the transcription of genes involved in cholesterol metabolism, through activation of the sterol regulatory element binding protein (SREBP).

However, this has been limited by undesirable off-target effects such as lipogenesis and elevated liver triacylglycerols, due to an increase of other SREBP-regulated genes [44]. Identifying and targeting a NAT that exerts discordant regulation over a gene such as erythropoietin (EPO), a hormone that stimulates bone marrow to produce red blood cells and is reduced in kidney disease, could be an alternative strategy to enhance its endogenous expression in order to treat dialysis-related anemia.

In addition, increasing or even partially restoring levels of growth factors that have been depleted due to disease pathology may also give rise to various protective effects. It has been demonstrated that oligonucleotide-mediated knockdown of individual NATs for human ephrin receptor B2 (EPHB2) and glial derived neurotrophic factor (GDNF) leads to a significant increase in sense gene expression, with up to four fold up-regulation seen for GDNF[19]. GDNF has been proposed as a treatment for Parkinson's disease, as experiment evidence suggests that it can be protective against the loss of dopaminergic neurons and even promote the regeneration of the nigrostriatal system in vivo [45]. Recently, Modarresi and colleagues also identified NATs for both mouse and human brain-derived neurotrophic factor BDNF [19], which has long been proposed as a therapeutic target for a range of neurodegenerative conditions, such as Alzheimer's disease (AD) [46] or Rett Syndrome [47]. For example, studies have shown that brain administration of a lentiviral vector constitutively expressing BDNF can not only rescue spatial and associative learning impairments in transgenic AD mice, aged rats and aged monkeys, but also significantly restored hippocampal synaptic function, protected against neuronal cell death and improved the observed amyloid-related disruption of gene expression [46]. Modarresi and colleagues demonstrated that siRNA-mediated knockdown of the antisense transcript for BDNF (BDNF-AS) in a human cell line resulted in substantial (up to four-fold) increase in both BDNF mRNA and protein levels. As with many other lncRNAs, BDNF-AS was observed to regulate BDNF transcription through the targeted recruitment of the histone lysine methyltransferase Ezh2, a core component of PRC2. Most significantly, the authors demonstrated that infusion of an antisense oligonucleotide (AntagoNAT) against BDNF-AS directly into the brains of wild-type mice not only resulted in an increase of BDNF mRNA and protein levels, but also induced neuronal outgrowth and differentiation. Taken together with GDNF and EphB2, this suggests a common type of discordant regulation across functionally related genes. Importantly, these findings, not only provide evidence that targeting NATs can be a viable therapeutic strategy to switch on gene expression *in vivo*, they also have considerable implications for the treatment of several neurodegenerative and neuropsychiatric disorders.

Another potential method to selectively de-repress gene expression is by blocking the interaction of NATs with their associated histone modifying enzymes, thereby inhibiting transcriptional silencing at their target loci. As discussed above, the ability to selectively block PRC2 activity could have benefits for the treatment of various types of cancer, by reactivating genes involved in tumor and metastasis suppression. Indeed in the previously described RIP-Seq study carried out by Zhao and colleagues, a large proportion of PRC2-associated RNAs corresponded to oncogene and tumor suppressor loci [48]. Furthermore, this strategy led to the identification of new PRC2-associated antisense transcripts for

HSP70 (protects neurons from protein aggregation and toxicity in numerous neurodegenerative diseases [49]), Malat-1 (promotes cancer metastasis [50]), Bgn (age-related osteoporosis [51]) and SCA2 (Parkinson's disease [52]), as well as RNAs linked to various other diseases. It is clear from this study that RNA-Seq and similar platforms could potentially be used to identify NAT/lncRNA-mediated interactions between PRC2 or other similar histone modifying complexes and therapeutically relevant gene loci.

Oligonucleotide strategies to activate gene expression, by targeting transcriptional silencing, could also be of benefit in the treatment of the many diseases arising from haploinsufficiency such as Dravet Syndrome, Soto's Syndrome etc [53, 54]. In these cases, various mutations or epigenetic phenomena can render one allele of a gene non-functional, resulting in reduced protein levels that are insufficient to maintain a normal cell phenotype. Therefore identifying and altering the activity of NATs that regulate such disease-related genes in a discordant manner could be a universal avenue to restore cellular protein expression to functional levels. Indeed, heterozygous deletion of the preproinsulin gene (INS) is a major risk factor for insulin-dependant diabetes, as the patient is left with only one functioning allele and insufficient levels of preproinsulin protein [55]. This strategy might also be a cost-effective alternative to the likes of enzyme replacement therapies for the treatment of other rare diseases.

Finally, another promising aspect of targeting NATs is that they could offer novel solutions to old problems. In the case of concordant regulation, manipulating NATs or other lncRNAs that promote the production or the cellular stability of a specific sense transcript could also be an effective method to indirectly reduce or normalize gene expression in various diseases. For example, the beta-site cleavage enzyme 1 (BACE1) has been an attractive therapeutic target for many years due its rate-limiting role in the production of amyloid beta (Aβ), a peptide fragment that is known to be pathogenic in Alzheimer's disease (AD). As such, a number of pharmaceutical companies have invested substantial time and resources into developing compounds that block BACE1 activity. This has been complicated however by various adverse effects, likely due to the other physiological functions of the enzyme [56]. Faghihi and colleagues have shown that a NAT for BACE1 (BACE1-AS) is elevated in the brains of patients with AD, as well as animal models for the disease. BACE1-AS was seen to improve the stability of BACE1 *in vitro*, by masking a regulatory microRNA binding site, thereby enhancing its cellular expression. Importantly, they also reported that infusion of siRNAs targeting BACE1-AS, directly into the brains of mice, not only decrease BACE1 mRNA and protein levels, but could also reduced soluble Aβ levels and Aβ aggregation patterns *in vivo*. Therefore, while completely blocking the activity of BACE1 can lead to problematic side effects, this type of indirect method to reduce BACE stability could possibly be used to normalize it cellular expression levels. Mahmoudi and colleagues have recently identified WRAP53, an antisense transcript that is over-expressed in a variety of cancer lines and regulates the p53 tumor suppressor gene in a concordant manner. Encouragingly, knockdown of WRAP53 induced apoptosis in cancer cells, which led the authors to propose that WRAP53 could represent a novel molecular target for the treatment of various malignancies [57]. In the future, as the number of functionally validated NATs continues to increases so too will the opportunities to manipulate disease-associated genes or

pathways that have otherwise been difficult to hit with more traditional pharmacological approaches.

Antisense oligonucleotides: the development of RNA-based therapeutics

The wealth of recent information regarding the involvement of RNAs in various diseases has led to a shift in focus towards designing more effective tools to modify their activity. By their very nature, ncRNA molecules are harder to influence by many currently available drugs, which traditionally target the protein products of genes, such as receptors, ion channels, enzymes etc. Fortunately, in the last few years there have also been a number of advances in antisense oligonucleotide technology. The design of second-generation oligonucleotide chemistries, such as phosphorothiote (PS) bonds and 2′-O′-methyl/2′O-methoxyethyl or locked nucleic acid (LNA) modifications, has led to the development of better-tolerated and more effective compounds. In addition, successful optimization of these secondary chemistries can ensure similar metabolic and toxicity profiles regardless of their target sequence. Further advantages of oligonucleotide-based therapeutics are low screening costs and longer half-lives, leading to simplified dosing regimens. Although few in number so far, some oligonucleotide-based drugs have already been approved for the treatment of conditions such as cytomegalovirus retinitis, and macular degeneration, with over thirty more in either phase 2 or phase 3 clinical trials.

It should be noted that, while this field continues to develop, a number of hurdles still remain, most notably the delivery of oligonucleotide-based therapies to the CNS. However advances are also being made in the design of more effective carriers for RNA-targeting therapies. For example there is evidence that positively charged cell-penetrating peptides (CPPs) [58] or liposomes [59] could be employed for successful delivery of DNA material, such as oligonucleotides, across the blood brain barrier. Furthermore, it will also be interesting to see whether the “Trojan Horse” approach, which has used nanoparticle-bearing macrophages to successfully deliver other molecules to the brain [60], could also be adapted for oligonucleotides.

Future directions

Although still in its infancy, the successful manipulation of natural antisense function *in vivo* has been a significant breakthrough, and clearly demonstrates the potential of this approach as a therapeutic strategy [19]. The recent creation of companies centered on long non-coding RNA-based therapies would also suggest a similar level of growing optimism within the scientific community. Clearly, just a very small fraction of the large new universe of long non-coding RNAs, notably antisense transcripts, has been pursued as drug targets thus far. As oligonucleotide approaches improve further, it is safe to predict that many long non-coding RNAs will be studied with such improved tools in academia as well as industry. An interesting future frontier will be the pursuit of small molecules that target long non-coding RNAs bound to chromatin regulating, as well as other, protein complexes.

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Tables 1

Recent examples of regulatory lncRNAs with potential roles in disease

	Gene	Disease	Reference
NATs	MALAT1	Cancer	[61]
	ATXN80S	Spinocerebellar ataxia	[62]
	ASFMR1	Fragile × syndrome	[63]
	NAT-RAD18	Alzheimer's disease	[42]
	PINK1-AS	Parkinson's disease, diabetes	[34]
	ANRIL	Cancer, cardiovascular disease, diabetes	[39]
	BACE1-AS	Alzheimer's disease	[18]
	NPPA-AS	Cardiovascular disease	[35]
	HTTAS	Huntington's disease	[33]
	p15AS	Cancer	[41]
LincRNA	HOTAIR	Cancer	[64]
	LincRNA-p21	Cancer	[65]
	LincRNA-EPS	Anemia	[66]
	LSINCT5	Cancer	[67]
	CUDR	Cancer	[68]

Table 2

Functional validation of natural antisense regulation *in vitro*.

	Sense	Designated Antisense	Species	Reference
Concordant Regulation	PINK1	PINK1-AS	Mouse, human	[34]
	BACE1	BACE1-AS	Mouse, human	[18]
	iNOS	iNOS-AS	Rat	[28]
	p53	Wrap53	Human	[57]
	HAS2	HAS2-AS	Human	[69]
	WDR83	DHPS	Human	[70]
Discordant Regulation	Msx-1	Msx-1 As	Mouse	[71]
	HIF1-a	aHIF1	Human	[31]
	Crx	CrxOS	Mouse	[72]
	D5D	Reverse D5D	Human	[73]
	Rad18	Nat-Rad18	Rat	[42]
	p15	p15AS	Human	[41]
	BOK	BOK-AS	Human	[74]
	NPPA	NPPA-AS	Human	[35]
	SLC39A5	ANKRD52	Human	[16]
	CCPG1	CCPG1-AS	Human	[16]
	RAPGEF3	RAPGEF3-AS	Human	[16]
	Tie-1	hAS-2	Human	[75]
	BDNF	BDNF-AS	Mouse, human	[19]
	GDNF	GDNF-AS	Human	[19]
	EPHB2	EPHB2-AS	Human	[19]
	Tbca13	Tbca16	Human	[76]
	Tspo	Tspo-Nat	Mouse	[77]